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For a tumor to metastasize it has to invade the surrounding tissue and evolve from a ductal carcinoma in situ to an invasive tumor, and angiogenesis is a key step. Metastatic and invasive breast cancers have perturbed stromal-epithelial interactions and changes in integrin expression (the receptors for the extracellular matrix-ECM-), particularly α5. Thus we hypothesized that increased expression of provisional integrins such as αv, α5, β1 and β6, as induced by the reactive stromal ECM, compromises mammary tissue organization to induce a pro-angiogenic and invasive phenotype in mammary epithelial cells. Using the HMT3522 breast cancer progression cell series we found that malignancy correlates with loss of tissue structure, expression of αv and α5 integrins and pro-angiogenic ability in co-cultures with endothelial cells, and increase in VEGF and Il-8 expression; while phenotypic reversion of the tumors correlates with loss of the angiogenic phenotype and downregulation of αv and α5 integrin expression. This same effect can be achieved by blocking α5 integrin. Finally, upregulation of α5 integrin in S3 cells (which are already angiogenic, but express lower levels of Il-8) induced a malignant phenotype in vitro, shown by invasion and growth in soft agar. *In vivo* assays are underway to confirm this.

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Table of Contents

2004 Progress report for Award Number DAMD17-03-1-0421
Principal Investigator: **Rozenberg, G.I.**

Introduction:

Stromal-epithelial interactions regulate mammary gland development and homeostasis (1). The extracellular matrix (ECM; which is the protein component produced by the stromal cells in the mammary gland) modulates mammary epithelial cell (MEC) growth, apoptosis (death) and differentiation in culture and *in vivo* (2) via transmembrane receptors called integrins, and metastatic and invasive breast cancers are characterized by perturbed stromal-epithelial interactions and changes in integrin expression (3). Moreover, correcting the integrin perturbations found in immortalized mammary tumor cells is sufficient to repress expression of the malignant phenotype in culture and reduce tumorigenicity in vivo (4,5). While angiogenesis, the growth of new blood vessels, is an essential step in tumor progression and metastasis formation (6,7), it is still not really clear what drives the angiogenic switch that will allow a benign lession like a DCIS to evolve to an invasive tumor and finally metastasize. Key pro angiogenic regulators secreted by tumor cells include vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGFs), and major antiangiogenic molecules produced by tumors include the thrombospondins (Tsp). The prevailing hypothesis has been that angiogenesis is primarily controlled by genetic factors that synergistically influence the tumor cells to induce expression of pro angiogenic molecules such as VEGF by driving expression of regulators such as HIF-1a (8-10) or anti angiogenic molecules such as Tsp. But this hypothesis does not sufficiently explain why DCIS lesions, which are not hypoxic, frequently exhibit a profound angiogenic phenotype (11) nor adequately explain the phenomenon of tumor dormancy in which tumors can reside in hypoxic environments for years without inducing angiogenesis. We, and others have noted that the altered 'reactive' stroma surrounding the tumor might have either direct or indirect effects on angiogenesis (12-15) via eliciting indirect effects on the epithelial cells. *Thus, our hypothesis is that because regulated stromal-epithelial interactions determine tissue organization and polarity by regulating cell adhesion, loss of tissue structure induced by a reactive stroma by perturbing cell adhesion is in term what pushes the pre-malignant cells to express a pro-angiogenic phenotype, thereby driving malignant transformation of DCIS lesions.*

Body:

Over the course of the last year I've made significant progress on all three of my specific aims.

- My first specific aim was to *determine if there was a correlation between tumorigenicity, loss of acinar structure, αv and/or α5 integrin expression and angiogenesis.*
- My second aim was to *test if the reacquisition of a polarized acinar structure is related to the loss of the angiogenic phenotype and if this is linked to changes in αv and/or α5 integrin expression.*
- My third aim was to *determine if the upregulation of αv and α5 integrins is a predictor of malignant behavior in HMT-3522 MECs, and functions by compromising tissue organization and inducing angiogenesis and invasion.*

Progression:

The first thing I did was to establish a new epithelial-endothelial cell co-culture model that was actually better correlated to in vivo than the one presented as preliminary data in my grant proposal. Instead of working with BAECs (Bovine aortic endothelial cells) I have been working with HUVECs (Human umbilical vein endothelial cells) and HDMVECs (Human dermal microvascular endothelial cells), which are both of human origin. I managed to grow these cells and establish co-cultures with MECs (Fig 1)

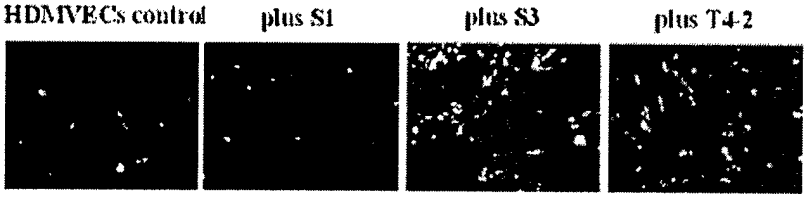

Fig1: HDMVECs pre-stained with PKH26 red fluorescent linker that invaded the collagen layer. Left: No MECs. Plus S1, S3 or T4-2: co-cultured with normal, premalignant or tumor MECs respectively.

MECs were grown in 3D reconstituted basement membrane (rBM) -Matrigel- in transwells for 10-12 days and then these transwells were transferred to another plate containing HDMVECs grown to 80% confluency and over-layered with collagen I. There was no significant angiogenic response with a normal tissue architecture (S1 cells), while the tumor cells induced a very robust angiogenic response (T4-2) (Fig

1). Surprisingly, the premalignant cells (S3) induced significant angiogenesis, although it was not as profound as the response induced by the tumors. This drove me to ask whether or not this correlated with changes in the expression of proangiogenic factors by the normal, premalignant and tumor cells. I did ELISAs (Enzyme linked immunosorbent assays) for the most common angiogenic molecules and, consistent with my preliminary data by RT-PCR and the co-culture model, the normal S1 cells expressed very low levels of VEGF (Fig 2 A). However, the premalignant cells already expressed very high levels of VEGF, similar to the ones secreted by the tumor cells. This is consistent with the fact that they do induce angiogenesis in co-cultures. However, they do not seem to have as strong an effect as the tumor cells. This led me to study other proangiogenic molecules. While levels of bFGF were very low in all of the studied cell lines (Fig 2 B), I found that it was actually interleukin 8 (Il-8) expression that was being upregulated from the normal to the premalignant to the tumor cells (Fig2 C).

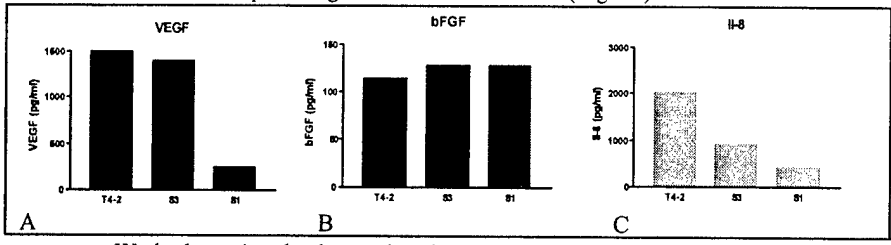

Fig 2: Expression of proangiogenic molecules by conditioned media form 3D rBM cultures of MECs. A) VEGF; B) bFGF; C) Il-8

We had previously shown that the tumor phenotype can be reverted by blocking β1 integrin (5), recapitulating a normal tissue structure. Then, I wanted to assess if the tissue structure was affecting the angiogenic response. As expected, phenotypic reversion of the tumors, induced downregulated expression of both VEGF and Il-8 (Fig 3).

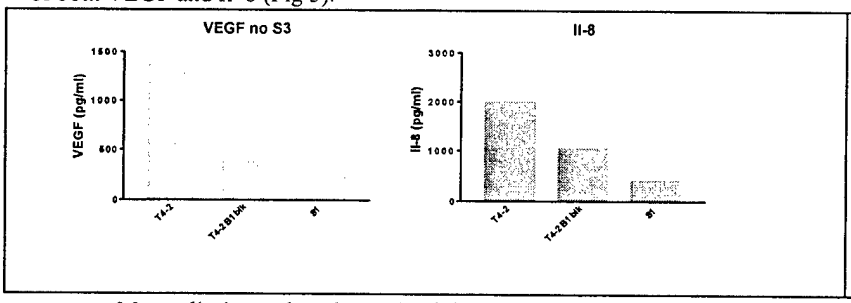

Fig 3: Phenotypic reversion downregulates VEGF and Il-8 expression. Expression of proangiogenic molecules in conditioned media form 3D rBM cultures of MECs +/-β1 integrin blocking antibody

My preliminary data determined that phenotypic reversion was associated with α5 and αv integrin protein downregulation. My new data confirms this, since by blocking α5 integrin in the tumor cells reverted morphological features, decreasing colony size and inducing loss of polarization; and also impaired tumorigenicity in vitro (anchorage independent growth in soft agar) (Fig 4).

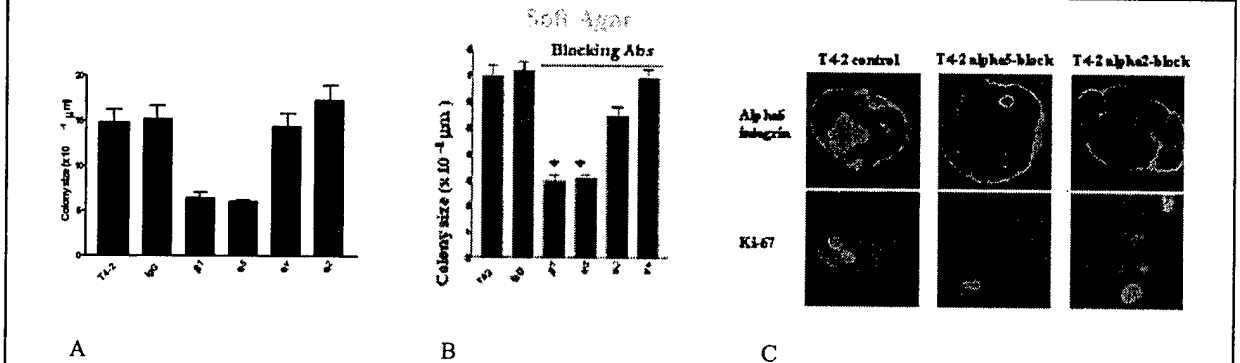

Fig 4: Phenotypic reversion of the tumors by β1- and α5-blocking but not α2- or αv- integrin blocking. A) Colony size after 12 days in 3D rBM in the presence of different blocking antibodies. B) Colony formation in soft agar after 21 days in the presence of different blocking antibodies. C) Immunostaining of 12 days colonies in 3D rBM showing reversion (restoration of polarity and proliferation control) by α5 integrin- blocking but not α2 integrin blocking.

2004 Progress report for Award Number DAMD17-03-1-0421
Principal Investigator: **Rozenberg, G.I.**

I have shown that phenotypic reversion of the tumors can be achieved by blocking α5 integrin, and that β1 blocking can tamper VEGF expression. Whether blocking α5 integrin is sufficient to revert the angiogenic phenotype in the co-culture model, and if it is associated with the expression of proangiogenic molecules, as well as if blocking α5 integrin will impair tumorigenicity *in vivo,* we don 't know. Thus now I have studies in progress to try to answer these questions.

The difficulty of course for the *in vivo* and even for the co-culture model is that an integrin blocking antibody could also have effects on the endothelial cells themselves, since α5 integrin directly affects endothelial cells growth and angiogenesis. I have therefore taken steps towards developing a siRNA to knock down α5 integrin mRNA expression. This, we hope, will definitely help us understand the effects of α5 integrin in malignant transformation.

Experiments are also underway to explore the role of αv integrin in tumor progression and angiogenesis. But because the effects on phenotypic reversion and anchorage independent growth and survival were not as evident as we noted for α5 integrin, these experiments have been temporarily delayed.

Regarding specific aim 3, I successfully transfected α5 and αv integrin subunits into the S1 and S3 cells, but this didn't really seem to have an effect on their phenotype in 3D rBM. In soft agar, I saw that S3α5 cells now acquired tumorigenic ability and grew significantly more than the control cells (Fig 5).

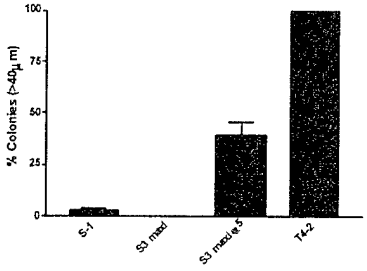

Fig 5: Anchorage independent growth and survival of MECs. Cells were grown in soft agar for 21 days and colony size was measured. Colonies were considered positive if they measured more than 40 μ

My next steps are to study if these correlates with effects on angiogenesis and tumorigenicity *in vitro* and *in vivo*.

In the case of the S1 cells, which do not express fibronectin (FN) in 3D (the ligand for α5β1 integrin), S1eGFP cells and S1-α5 cells were grown in 3D rBM for 12 days. Immunostaining for different polarity markers showed no difference between these cells, but the addition of exogenous FN made the α5 expressing cells lose polarity and give rise to bigger colonies in 3D (Fig 6).

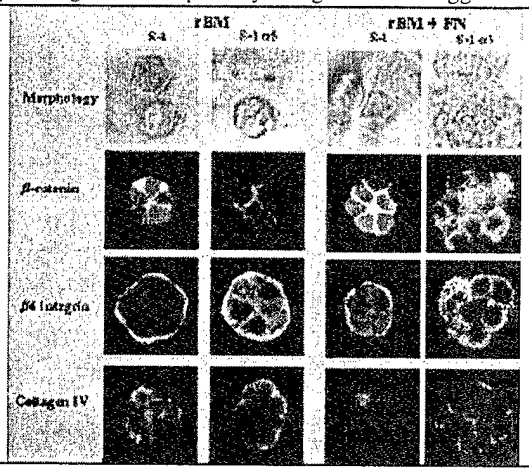

Fig 6:
Left panel: S1eGFP cells and S1-a5 cells were grown in 3D rBM for 12 days. Immunostaining for different polarity markers showed no difference between them.
Right panel: S1eGFP cells and S1-α5 cells were grown in 3D rBM plus FN for 12 days. Colony size measurements and immunostaining for different polarity markers showed that α5 integrin expression already had an effect on these nonmalignant cells.

Key Research Accomplishments:

- I have done expression characterization of protein by FACs Analysis in S-1, S-3 and T4-2 cells in 2D monolayer.

2004 Progress report for Award Number DAMD17-03-1-0421
Principal Investigator: **Rozenberg, G.I.**

- Transcriptional characterization of S-1, S-3 and T4-2 cells (as well as T4-2 revertants) in 3D rBM by microarray analysis is now in progress.
- I have completed the immunological characterization of S-1, S-3 and T4-2 cells in 3D rBM (Manuscript by Ritzki et al, to be submitted shortly).
- I have done protein expression characterization of the S-1, S-3 and T4-2 cells in 3D rBM (see fig 2)
- I have successfully set up the epithelial-endothelial cell co-culture assays.
- I have done angiogenesis characterization of S-1, S-3 and T4-2 cells in 3D multi-cellular cultures *ex vivo* (see Fig 1).
- *In vivo* experiments are now being started.
- I have done immunological characterization of T4-2 revertants in 3D rBM, for angiogenic potential and for phenotypic reversion using integrin blocking antibodies and this is still in progress. I have successfully demonstrated that phenotypic reversion can be achieved by blocking $\alpha 5$ integrin subunit in the tumor cells (see fig 4).
- Protein expression characterization of the T4-2 revertants is now in progress (also see fig 2)
- Angiogenesis characterization of the T4-2 revertants in co-cultures is now in progress.
- I have prepared $\alpha 5$ and αv integrin wild type and mutant retroviral inducible expression constructs and retroviruses.
- I have successfully prepared and characterized pooled populations of T4-2, S3 and S1 cells expressing $\alpha 5$ and αv integrins.
- I have done expression characterization of protein by FACs analysis in S-1 and S-3 cells expressing $\alpha 5$ integrin in 2D monolayer.
- I have done immunological characterization of S-1 and S-3 cells expressing $\alpha 5$ integrin in 3D rBM.
- I have preliminary data for the invasion characterization of S-1 and S-3 cells expressing $\alpha 5$ integrin.
- Angiogenesis characterization of the S-1 and S-3 cells expressing $\alpha 5$ integrin in co-cultures is now in progress.

Thus far, I have identified some good candidates that could be modulating malignant transformation that are pro angiogenic. I have also demonstrated that phenotypic reversion is not only associated with reorganization of normal architecture but that it downregulates proangiogenic molecules. Now, I need to examine: does that also drive down angiogenic response in the co-culture assay and does that also impair the angiogenic response in vivo?

Reportable outcomes:

Publications and Abstracts:

- "Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors." Zahir N, Lakins JN, Russell A, Ming W, Chatterjee C, Rozenberg GI, Marinkovich MP, Weaver VM. J Cell Biol. 2003 Dec 22;163(6):1397-407.
- "Identification of functionally significant changes in transition from premalignant to malignant phenotype". A. Rizki, V.M. Weaver, K.Chin, S-Y Moonlee, G. Rozenberg, C.A. Myers, J.L. Bascom, J.D. Mott, R.A. Jensen, O.W. Petersen, D.J. Chen, F. Chen, J.W. Gray, M.J. Bissell. (to be submitted shortly)
- "Fibronectin-ligated-$\alpha 5\beta 1$ integrin activates RhoA and PI3 Kinase to promote malignant behavior of mammary epithelial cells". Rozenberg, G.I., Lakins, J.N., Friedland, J. and Weaver, V.M. (In preparation)
- "Malignant behaviour of mammary epithelial cells depends on alpha5 beta1 integrin - fibronectin interactions". Gabriela Rozenberg, Jonathon Lakins, Chandrima Chatterjee and Valerie Weaver. 95th Meeting of the American Association for Cancer Research; Orlando, FL, USA. March 2004.
- "Fibronectin-alpha5 beta1 integrin interactions dictate the malignant behavior of mammary epithelial cells". G.I. Rozenberg, J.N. Lakins, C. Chatterjee and V.M. Weaver. 43rd Meeting of the American Association for Cancer Research; San Francisco, CA, USA. December 2003. (Oral presentation)
- "Identification of gene classes associated with transition from premalignant to malignant phenotype in human breast epithelial cells". A. Rizki, V.M. Weaver, K.Chin, S-Y Moonlee, K. Franks, G. Rozenberg, C.A. Myers, J.D. Mott, L.R. Grate, O.W. Petersen, R.A. Jensen, O.W.

Petersen, D.J. Chen, F. Chen, J.W. Gray, S. Mian and M.J. Bissell. 43rd Meeting of the American Association for Cancer Research; San Francisco, CA, USA. December 2003.

Conclusions:

Thus far I have established that malignant transformation and breast cancer behavior is associated with upregulation of α5 and αv integrin expression and with the acquisition of a proangiogenic phenotype, which is already seen in the premalignant cells. I have also shown that phenotypic reversion of the tumors correlates with downregulation of angiogenic factors, and that moreover, it appears that malignant transformation correlates not with an increase in VEGF expression but most probably with an increase in Il-8 expression, since the premalignant cells express high levels of VEGF but much lower levels of Il-8. I also showed that Il-8 expression is dowregulated with reversion of the tumor phenotype. In the next year I am planning on doing these co-culture assays with the reverted tumors, to see if this holds true. For this, our laboratory has developed an siRNA against β1 integrin, and I am also working on developing an siRNA for α5 intergrin.

I am also planning on setting up the *in vivo* assays to see if α5 integrin expression correlates with a proangiogenic and tumorigenic phenotype.

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